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Ca^{2+}-INDUCED CHANGES IN THE BARRIER PROPERTIES OF CARDIOLIPIN/PHOSPHATIDYLCHOLINE BILAYERS

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Summary

(1) A selective increase in permeability is induced in cardiolipin/phosphatidylcholine bilayers at Ca^{2+} concentrations of 1—3 mM. At higher concentrations of Ca^{2+} the permeability barrier is completely destroyed.

(2) The selective increase in permeability is correlated with the formation of lipid particles visualized by freeze-fracture electron microscopy and an isotropic signal in ^{31}P-NMR spectra.

(3) Lowering the Ca^{2+} concentration shows reduction in permeability but the formation of the lipid particles is a non-reversible process.

(4) At higher Ca^{2+} concentrations, ^{31}P-NMR spectra and freeze-fracture results indicate the formation of the hexagonal phase, explaining the disappearance of the permeability barrier.

Introduction

It is well established that a large part of the lipids in biological membranes is arranged in a lipid bilayer [1,2]. In isolated form, however, certain lipids have been shown to prefer nonbilayer configurations, such as the hexagonal (H_{II}) phase [3]. Recently, some model membranes have been described in which the lipid organization is a combination of bilayer and non-bilayer arrangements [4]. This type of organization was visualized by freeze-fracture electron microscopy as particles and pits associated with a lipid bilayer and detected by ^{31}P-NMR from the isotropic motion of the phospholipids [4]. Isotropic motion of phospholipids has also been detected by ^{31}P-NMR in rat, bovine and rabbit liver microsomes [5,6] and lipid particles have been observed

in freeze-fracture electron micrographs of total lipids extracts of *Escherichia coli* [7] and bovine rod outer segments [8]. It is an attractive hypothesis that such non-bilayer lipid structures in membranes may play a role in fusion and transport across the membrane [9].

One of the model membranes in which non-bilayer structures can be introduced consists of an equimolar mixture of bovine heart cardiolipin and egg yolk or 18 : 1_c/18 : 1_c-phosphatidylcholine [4]. In this system, lipid particles and isotropic motion of phospholipids are observed upon addition of Ca^{2+} [4]. As a model for this structure we proposed the inverted micelle, sandwiched in between the two monolayers of the lipid bilayer [4].

Using this model system, an enhanced transbilayer movement [10] of lipids and membrane fusion [11] could be related to the appearance of lipid particles. In the present study, we report on Ca^{2+}-induced permeability changes in single-walled vesicles and multilayered liposomes of such a phosphatidylcholine/cardiolipin mixture and the possible role of polymorphic phase transitions in this respect.

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine) and egg yolk phosphatidic acid were synthesized as described before [13]. Bovine heart cardiolipin was obtained from Sigma (St. Louis, U.S.A.), [^{3}H]dextran (M_r 77 500) and ^{86}RbCl from the Radiochemical Centre (Amersham, U.K.). All other chemical reagents were of analytical reagent grade. Lipid mixtures were evaporated to dryness and further dried overnight under vacuum. To prepare liposomes a lipid film was dispersed in Tris-buffered KCl solution as described previously [14]. Unilamellar vesicles (etherosomes) were prepared according to the ether-evaporation method described by Deamer and Bangham [12]. The lipids were dissolved in ether to a concentration of 2 mM and 2.3 ml of this solution were injected into 4 ml Tris-buffered KCl solution at 55°C. The dispersion was centrifuged at 7000 × *g* during 30 min to remove a lipid fraction of fused vesicles. Subsequent centrifugation at 200 000 × *g* during 60 min delivered a fraction of pure single-walled vesicles as judged by freeze-fracture electron microscopy. The etherosomes were resuspended and dialyzed overnight at room temperature against Tris-buffered KCl to remove traces of ether.

K^+ efflux was measured with a combination of a K^+ selective glass electrode (Philips, No. G 15 K) and a reference electrode (Philips, No. R44/2 SD 1), both connected to a pH 26 Radiometer (Copenhagen, Denmark), after replacing non-trapped KCl by isotonic choline chloride, either by repeated centrifugation or by intensive dialysis at 4°C. Trapped K^+ was released by addition of Triton X-100. Ca^{2+} was added to the liposomes either by addition of an aliquot of a 1 M $CaCl_2$ solution or by suspending the liposomal pellets in Tris-buffered isotonic choline chloride containing the required Ca^{2+} concentration.

High-power proton noise-decoupled ^{31}P-NMR spectra were recorded at 36.4 MHz as described before [15]; a sweep width of 12 kHz and a pulse rate of 0.17 s employing 45° radio frequency were used. To enhance the signal-to-noise ratio, the free induction decay in these experiments was

multiplied by an exponential function resulting in a 50 Hz line broadening.

Freeze-fracture electron microscopy was performed as outlined previously [16]; glycerol was added to the samples to prevent freeze damage.

Results and Discussion

When Ca^{2+} is added to a suspension of KCl-filled unilamellar etherosomes of an equimolar mixture of bovine heart cardiolipin and 18 : 1_c/18 : 1_c-phosphatidylcholine in a choline chloride medium, a Ca^{2+}-induced K^+ permeability can be measured. As shown in Fig. 1, concentrations of up to 3 mM Ca^{2+} have only a moderate effect, whereas above this concentration a very rapid release is obtained. Also, in the experiments with multilayered compartment systems,

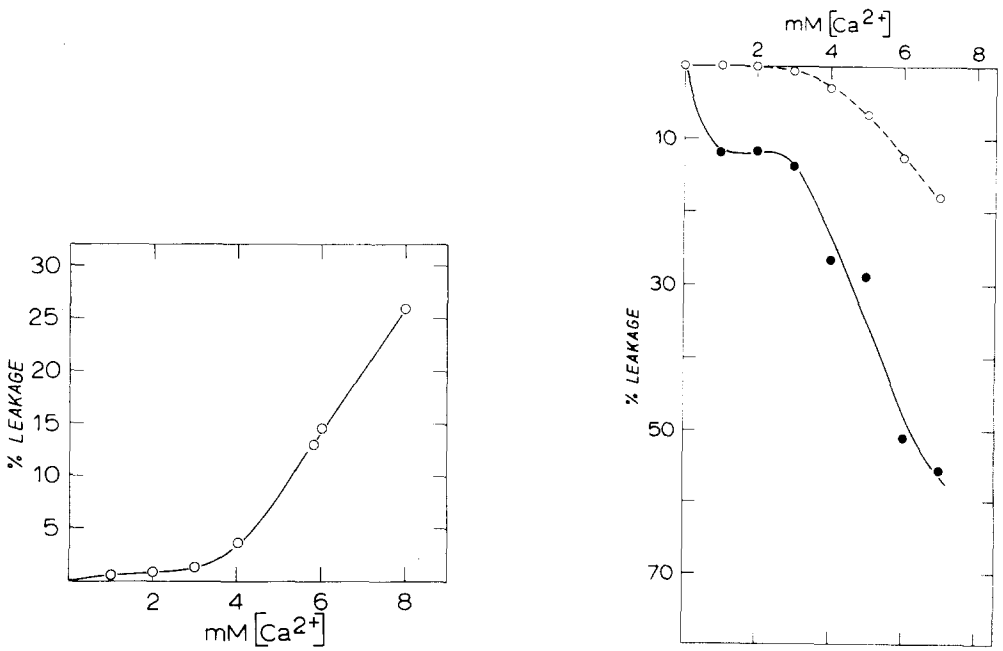

Fig. 1. Ca^{2+}-induced efflux of K^+ from cardiolipin/phosphatidylcholine on single-walled vesicles (etherosomes). Etherosomes of bovine heart cardiolipin and 18 : 1_c/18 : 1_c-phosphatidylcholine (1 : 1) were prepared in 150 mM KCl, 0.2 mM EDTA, 10 mM Tris/acetic acid, pH 7.0, and dialyzed against isotonic choline chloride. Small aliquots of the etherosomes were dispersed in 5 ml choline chloride solution and by addition of 1 M $CaCl_2$ the Ca^{2+} concentration was adjusted to the desired values. The K^+ efflux was measured as a function of time using a K^+-sensitive electrode. The data given are the amounts of Ca^{2+}-induced K^+ release during the first 5 min after Ca^{2+} addition.

Fig. 2. Ca^{2+}-induced release of K^+ (●) and [^{3}H]dextran (○) from liposomes of bovine heart cardiolipin and 18 : 1_c/18 : 1_c-phosphatidylcholine (1 : 1), recorded after 90 min at 23°C. Release of K^+ was measured from liposomes prepared in 150 mM KCl, 0.2 mM EDTA, 10 mM Tris/acetic acid, pH 7.0, sedimented at 37 000 × *g* for 30 min and dispersed in isotonic choline chloride containing the indicated concentrations of $CaCl_2$ (lipid concentration 4 mM). After incubation for 90 min at 23°C, the released and still-trapped K^+ were measured as described before. Release of [^{3}H]dextran was measured from liposomes prepared in 150 mM KCl, 0.2 mM EDTA, 10 mM Tris/acetic acid, pH 7.0, containing 5.0 μCi/ml [^{3}H]dextran. After sedimentation at 37 000 × *g* at 30 min the liposomes were washed three times with dextran-free buffer, dispersed in buffer containing the indicated concentration of $CaCl_2$ and incubated for 90 min at 23°C. After centrifugation the supernatants were assayed for [^{3}H] radioactivity.

a significant but slow release of K^+ at low Ca^{2+} concentrations and a rapid increase in rate above 3 mM Ca^{2+} can be observed. The moderate K^+ leakage from etherosomes at low Ca^{2+} concentrations is linear for a long period of time, whereas the comparable leakage from multilayered structures, initially of the same rate as that from the etherosomes, decreases after some time. This gives rise to a constant level in the extent of leakage from the multilayered liposomes in the [Ca^{2+}] range from 1 to 3 mM when this is measured after 90 min incubation time (cf. Fig. 2).

It is important to mention that under the conditions used, both the rate and extent of K^+ release are more related to the Ca^{2+} concentration than to the Ca^{2+} : cardiolipin ratio. In experiments using a 10-fold higher Ca^{2+} : lipid ratio, we obtained essentially the same results as in that given in Fig. 2. In control experiments with liposomes of 18 : 1_c/18 : 1_c-phosphatidylcholine and 4 mol% of egg yok phosphatidic acid, no Ca^{2+}-induced K^+ leakage was noticed.

These experiments indicate that Ca^{2+} affects the barrier function of a lecithin/cardiolipin bilayer. At higher concentrations, a drastic reorganization is brought about, enabling not only the passage of K^+ but also of Ca^{2+} which apparently penetrates the outer bilayer barrier at a rate sufficient to attack the inner bilayers of the multilayered liposomes as well. At low Ca^{2+} concentrations, a more selective K^+ permeability is obtained and the constant level of K^+ release, which is reached in the 1—3 mM Ca^{2+} range, can be explained as an exhaustion of the outermost water compartment. In this connection it can be argued that the size of this outer compartment wil be considerably larger than the 12% trapped volume which can be deduced from the horizontal level in the Ca^{2+}-dependent K^+ leakage of Fig. 2. The pretreatment of the sample already causes K^+ release from the outermost compartment and in the calculations of the net Ca^{2+}-induced K^+ leakages, notable blanks are substracted.

The induction of selective permeability is further supported by leakage experiments with [^{3}H]dextran. In the range 1—3 mM Ca^{2+}, where a significant Ca^{2+}-induced K^+ leakage can be measured, there is no permeability for [^{3}H]-dextran, whereas at higher Ca^{2+} concentrations also release of this large non-electrolyte molecule can be observed.

It is likely that the release at high Ca^{2+} concentrations is due to transformation of the bilayer structure into a hexagonal organization of the lipid molecules. The formation of a Ca^{2+}-induced hexagonal phase, next to molecular orientations allowing isotropic motion of the lipid molecules, has been reported for mixtures of cardiolipin and egg lecithin on the basis of freeze-fracture electron micrographs and typical ^{31}P-NMR spectra [4]. We could confirm these observations for the present lipid mixture when Ca^{2+} concentrations exceeding 3 mM were applied; the amount of hexagonal phase as estimated from the ^{31}P-NMR spectra was found to increase with increasing Ca^{2+} concentration.

Selective K^+ permeability in the range 1—3 mM Ca^{2+} must be the consequence of a more subtle rearrangement in the bilayer barrier. From our present knowledge different suggestions can be made in this respect. Firstly, it is conceivable that Ca^{2+} induces a lateral phase separation in the bilayer as has been shown for some mixtures with other negatively charged phospholipids [17]. There are several reports indicating that the existence of phase bounda-

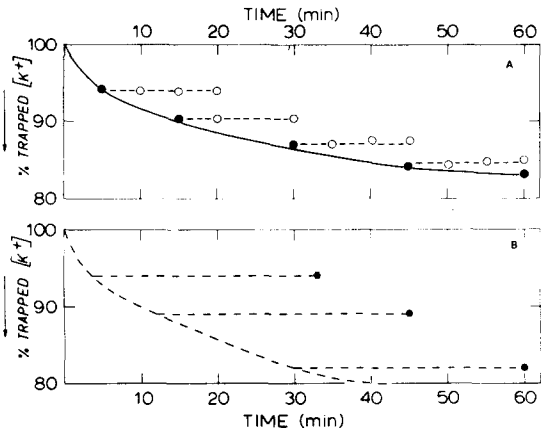

Fig. 3. Reversibility of the Ca^{2+}-induced permeability of cardiolipin/phosphatidylcholine liposomes to K^+ and Rb^+. Liposomes of bovine heart cardiolipin and 18 : 1_c/18 : 1_c-phosphatidylcholine (1 : 1) were prepared in 150 mM KCl, 0.2 mM EDTA, 10 mM Tris/acetic acid, pH 7.0, without or with 10 μCi/ml $^{86}Rb^+$ and sedimented at 37 000 X *g* for 30 min. Pellets were dispersed in isotonic choline chloride and KCl, respectively, either with or without 2.5 mM $CaCl_2$ (lipid concentration 4 mM), and incubated at 23°C. (A) At intervals, 0.2 ml samples were transferred into 5 ml of Ca^{2+}-free isotonic choline chloride (●———●) and further incubated for 15 min (○———○). Release of K^+ was determined as described before. (B) At intervals, samples were diluted 25-fold with Ca^{2+}-free buffer and further incubated at 23°C fo 30 min. Release of $^{86}Rb^+$ was determined by measuring supernatant radioactivity after sedimentation at 37 000 X *g*. In A and B the values of Ca^{2+}-induced release were corrected for the corresponding blanks.

ries in the plane of the bilayer can cause a selective increase in permeability [18,19]. However, we have no indication that such phase boundaries can be formed in the mixture of 18 : 1_c/18 : 1_c-phosphatidylcholine and the very unsaturated cardiolipin. On the other hand, Ca^{2+} concentrations up to 3 mM already change the ^{31}P-NMR spectrum of the liposomes of this lipid mixture. As shown in Fig. 4, the lipid molecules become able to undergo isotropic motion but at these low concentrations there is not sign that also hexagonal orientations are formed. The freeze-fracture pictures show the formation of particles which, as discussed in the Introduction, we explain as inverted micelles sandwiched in between the two monolayers of the lipid bilayer [4]. It is of interest that in the freeze-fracture pictures, next to fracture faces carrying the particles, fracture faces can be observed which are completely free of particles.

This result may indicate that in a number of bilayers the particles disappear during the freezing process or that Ca^{2+} induces particles in a selected number of bilayers, e.g., only in the outermost bilayers of the liposomes. Unfortunately, the freeze-fracture pictures as shown in Fig. 4 give insufficient support to conclude that the particle-free fracture faces originate from the inner bilayers of the multilayered structures.

The relationship between Ca^{2+}-induced selective K^+ permeability, the appearance of lipidic particles and an 'isotropic' ^{31}P-NMR signal was further investigated by studying the reversibility of these phenomena. In Fig. 3A is shown that the K^+ efflux from liposomes, induced by incubation with 2.5 mM Ca^{2+}, is stopped by dilution with buffer to a Ca^{2+} concentration of 0.1 mM. As, theoretically, this could be due to the inability of the large choline ions to

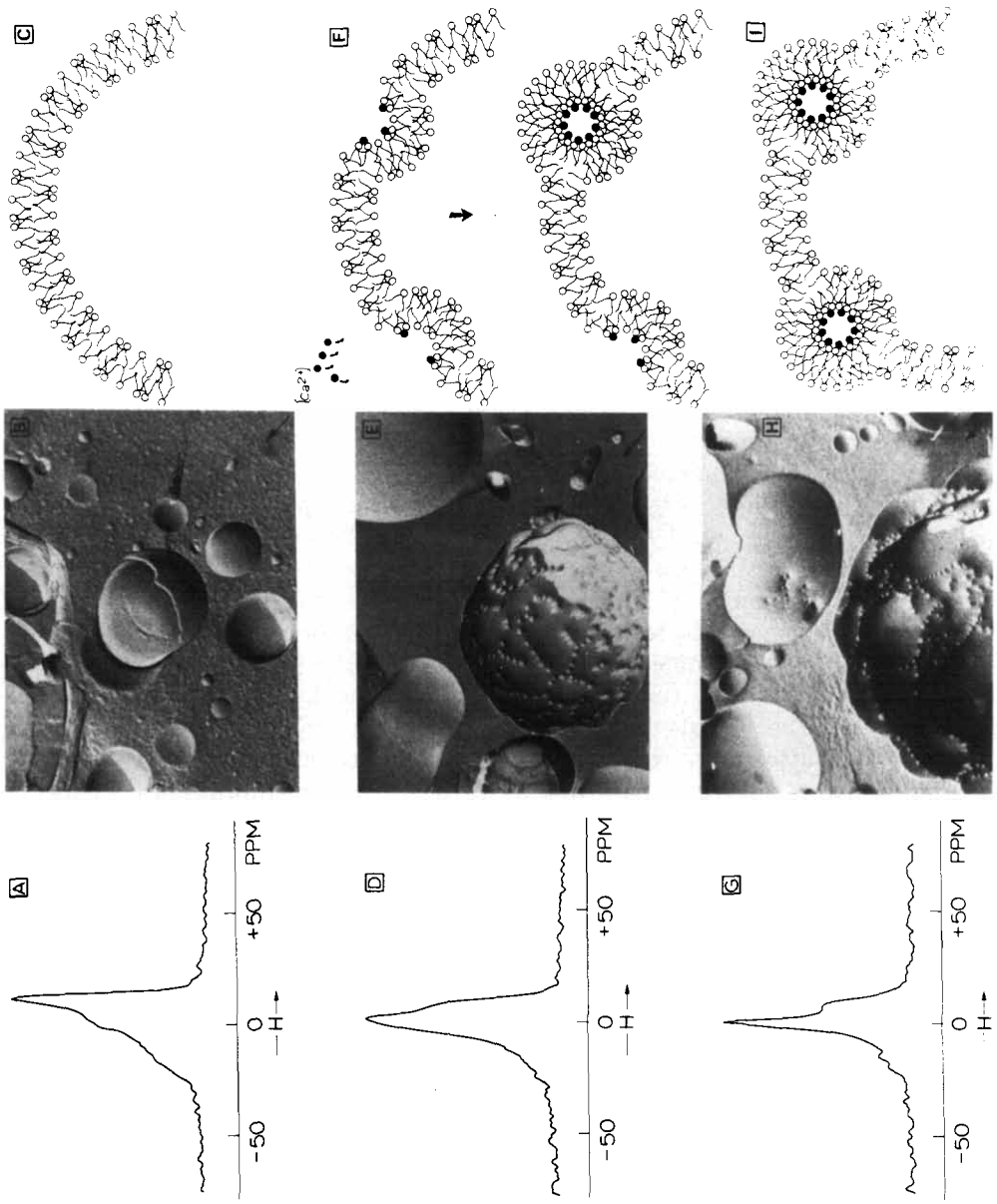

Fig. 4. Ca^{2+}-induced structural change in cardiolipin/phosphatidylcholine liposomes: ^{31}P-NMR and freeze-fracture electron microscopy. Liposomes of bovine heart cardiolipin and 18 : 1_c/18 : 1_c-phosphatidylcholine (1 : 1) were prepared in 150 mM KCl, 0.2 mM EDTA, 10 mM Tris/acetic acid, pH 7.0, and sedimented at 37 000 × *g* for 30 min. After recording the ^{31}P-NMR spectrum (A), the liposomes were sedimented and dispersed in isotonic choline chloride containing 2.5 mM Ca^{2+} (lipid concentration 4 mM) and incubated at 23°C for 90 min. After sedimentation, the ^{31}P-NMR spectrum (D) was recorded in the presence of 2.5 mM Ca^{2+}. Subsequently, the liposomes were dispersed in a 25-fold volume of choline chloride and sedimented again, after which the ^{31}P-NMR spectrum (G) was recorded. At the three stages, samples were withdrawn for freeze-fracture electron microscopy (B, E and H). The models (C, F and I) intend to show schematically the changes in the lipid organization. (Magnification, × 40 000. The arrows indicate the direction of shadowing.)

exchange with K^+ over the membrane, a comparison was made between the release of $^{86}Rb^+$ from liposomes in Tris-buffered KCl and the release of K^+ from liposomes in Tris-buffered choline chloride, both in the presence of 2.5 mM Ca^{2+} and after dilution to 0.1 mM Ca^{2+}. Fig. 3B shows that essentially the same results were obtained. Similarly to the release of K^+, the release of $^{86}Rb^+$ is also stopped by lowering the Ca^{2+} concentration from 2.5 to 0.1 mM. Therefore, it can be concluded that the induced K^+ permeability is a reversible process; removing Ca^{2+} stops the K^+ permeability. On the other hand, lowering the Ca^{2+} concentration did not cause the disappearance of the lipid particles observed by freeze-fracture electron microscopy or the 'isotropic' ^{31}P-NMR signal, although a small shift in the width of the signal is noticeable (Fig. 4G and H). This indicates that the increased permeability to K^+ is not caused by the existence of these structures through which the phospholipids undergo isotropic motion, but rather by the molecular motion leading to formation of the particles.

At present, our knowledge about the mechanism by which the particles are formed is limited. Fig. 4 illustrates one of the possible mechanisms by which the particles could be brought about. On the other hand, the particles could also arise from a Ca^{2+}-induced fusion between the surrounding bilayers of different liposomes [11]. The fusion process may be accompanied by dynamic rearrangements leading to increased membrane permeability, with formation of inverted micelles at the fusion sites. The irreversibility of particle formation suggests that the inverted micelle in the hydrophobic core of the bilayer is a rather stable organization. This does not support the idea that dynamic formation and resolution of the inverted micelles could act as an intrinsic carrier mechanism in which K^+ passes the membrane by enclosure in the hydophilic centre of the inverted micelle, although in the presence of Ca^{2+} such a mechanism may occur. In agreement with these observations, we have noticed that the existence of lipid particles in bilayers of the system, phosphatidylethanolamine/phosphatidylcholine/cholesterol, does not increase K^+ and glucose permeability [20].

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